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Ceftriaxone acts synergistically with levofloxacin in experimental meningitis and reduces levofloxacin-induced resistance in penicillin-resistant pneumococci

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Ceftriaxone acted synergistically with levofloxacin in time–killing assays *in vitro* over 8 h against two penicillin-resistant pneumococcal strains (WB4 and KR4; MIC of penicillin: 4 mg/L). Synergy was confirmed with the chequerboard method, showing FIC indices of 0.25. In the experimental rabbit meningitis model, ceftriaxone (1× 125 mg/kg) was slightly less bactericidal (–0.30 $\Delta\log_{10}$ cfu/mL·h) compared with levofloxacin (–0.45 $\Delta\log_{10}$ cfu/mL·h) against the penicillin-resistant strain WB4. The combination therapy (levofloxacin and ceftriaxone) was significantly superior (–0.64 $\Delta\log_{10}$ cfu/mL·h) to either monotherapy. In cycling experiments *in vitro*, the addition of ceftriaxone at a sub-MIC concentration (1/16 MIC) reduced levofloxacin-induced resistance in the two strains KR4 and WB4. After 12 cycles with levofloxacin monotherapy, the MIC increased 64-fold in both strains versus a 16-fold increase with the combination (levofloxacin + ceftriaxone 1/16 MIC). In both strains, levofloxacin-induced resistance was confirmed by mutations detected in the genes *parC* and *gyrA*, encoding for subunits of topoisomerase IV and gyrase, respectively. The addition of ceftriaxone suppressed mutations in *parC* but led to a new mutation in *parE* in both strains.

Keywords: *Streptococcus pneumoniae*, quinolones, β-lactam antibiotics

Introduction

The worldwide increase in penicillin-resistant strains has complicated the treatment of pneumococcal infections. In the USA, resistance rates have reached 51%, with 33% of the strains showing intermediate resistance in recent years.[1] A recently published survey from Switzerland also revealed an increasing tendency towards resistance, with overall rates of 13%.[2] Additional resistance to cephalosporins in some cases has further jeopardized the usefulness of β-lactam antibiotics in pneumococcal diseases. In addition, quinolone-resistant strains have been isolated.[3] The report of quinolone treatment failure due to the emergence of quinolone resistance during treatment has thrown the role of quinolone monotherapy in pneumococcal diseases into question.[4] Until now, β-lactam antibiotics have remained the drugs of choice for pneumococcal diseases, except when their penetration into infected tissues is limited, as is the case in meningitis. Published guidelines advise a combination of a cephalosporin with vancomycin for the empirical treatment of meningitis, especially when cephalosporin-resistant strains are suspected.[5] On the other hand, the recent isolation of cephalosporin- and vancomycin-tolerant strains might lead to eradication failures and reduce the utility of this antibiotic combination.[6] A highly bactericidal antibiotic combination with excellent tissue penetration that does not lead to the emergence of resistance would be a major advantage in the treatment of pneumococcal diseases. For more than a decade, ceftriaxone has been the established monotherapy for these infections. The aim of this study was to investigate the potential synergy between ceftriaxone and levofloxacin, both with good activity against pneumococci, *in vitro* and in experimental meningitis and to test the effect of ceftriaxone on levofloxacin-induced resistance *in vitro*.

Methods

Strains and MIC determination

The two pneumococcal strains (WB4 and KR4) were originally isolated from two patients with pneumonia at the University Hospital of Bern, Switzerland. The MICs for WB4 were: penicillin 4 mg/L, ceftriaxone 0.5 mg/L, cefotaxime 1 mg/L, vancomycin 0.12–0.25 mg/L, levofloxacin 1 mg/L, gatifloxacin 0.12–0.25 mg/L, moxifloxacin 0.12 mg/L and garenoxacin 0.03 mg/L. The MICs for KR4 were: penicillin 4 mg/L, ceftriaxone 0.5 mg/L, cefotaxime 1 mg/L, vancomycin 0.12–0.25 mg/L,

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levofloxacin 1 mg/L, gatifloxacin 0.25 mg/L, moxifloxacin 0.12 mg/L and garenoxacin 0.015 mg/L.

MICs were determined by broth macrodilution. The MIC was defined as the lowest concentration that inhibited visible growth after 12 and 24 h of incubation at 37°C.

Rabbit meningitis model

The meningitis model described by Dacey & Sande[7] was used in this study. The experimental protocol was accepted by the local ethics committee (Veterinäramt des Kantons Bern). Young New Zealand white rabbits weighing 2–2.5 kg were anaesthetized by intramuscular injection of ketamine (30 mg/kg) and xylazine (15 mg/kg) and were immobilized in stereotactic frames for induction of meningitis and cfu sampling. An inoculum containing ~10^5 cfu of the penicillin-resistant strain WB4 was instilled in the cisterna magna. A long-acting anaesthetic drug [ethylcarbamate (urethane): 3.5 g/rabbit] was injected subcutaneously, and animals were returned to their cages. Fourteen hours later the cisterna magna was punctured again for periodic CSF sampling before and 1, 2, 4, 5, 6 and 8 h after initiation of therapy. Anaesthesia was performed by repeat injections of pentobarbital sodium (Nembutal). Antibiotics were administered via a peripheral ear vein at the following concentrations: ceftriaxone 125 mg/kg and levofloxacin 10 mg/kg of body weight. Ceftriaxone and levofloxacin were injected once at hour zero. All antibiotics and anaesthetic drugs were purchased commercially.

Bacterial titres were measured by 10-fold serial dilutions of CSF samples, then plated on blood agar plates containing 5% sheep blood, and incubated overnight at 37°C. In parallel, 20 μL aliquots of undiluted samples were plated (limit of detectability: 50 cfu/mL). Comparisons between dilutions of CSF were used to exclude significant carryover effects during therapy. The antimicrobial activity of the different regimens during the 8 h treatment was calculated by linear regression analysis and expressed as a change in $\log_{10}$ cfu/mL·h and as a change in viable count over 8 h. A value of 1.7 ($\log_{10}$ of the limit of detectability) was assigned to the first sterile CSF sample, and a value of zero was assigned to any subsequent sterile CSF sample. The results are expressed as means ± S.D. Statistical significance was determined by the Newman–Keuls test.

Determination of antibiotic levels in CSF

Antibiotic concentrations in the CSF were measured by agar diffusion. Standard curves were performed in saline with 5% rabbit serum in order to mimic CSF protein concentrations.[8] *Bacillus subtilis* ATCC 6633 was used as a test strain for levofloxacin[9] and *Escherichia coli* (ATCC 25922) for ceftriaxone.[10] The inter- and intraday variability was in the range 5%–10%. The limits of detection were 0.5 mg/L for ceftriaxone and 0.3 mg/L for levofloxacin.

In vitro killing assays

The two pneumococcal strains (WB4 and KR4) were grown in C+Y[11] to an optical density of 0.3 at 590 nm and then diluted 40-fold to 10^6 cfu, corresponding approximately to the CSF bacterial titre in rabbits before initiation of therapy. Ceftriaxone was added at a sub-MIC concentration (1/2 MIC against KR4 and WB4) and levofloxacin at the concentration corresponding to the MIC. Bacterial titres were determined at 0, 2, 4, 6 and 8 h by serial dilution of samples, plated on agar plates containing 5% sheep blood and incubated at 37°C for 24 h. Experiments were performed in triplicate and results are expressed as means ± S.D.

Determination of fractional inhibitory concentration (FIC) index

FIC indices were measured using the chequerboard method, as described previously.[12] In brief, the two pneumococcal strains (WB4 and KR4) were grown in C+Y medium until the logarithmic growth phase (optical density of 0.3 at 590 nm) and were then diluted 1:40. Approximately 0.5–1 × 10^6 cfu were pipetted into microtitre trays containing concentrations of levofloxacin and ceftriaxone that ranged from 1/32 × MIC to 2 × MIC. Microtitre plates were incubated at 37°C for 24 h. After 6, 12 and 24 h the plates were read for detection of inhibition of bacterial growth. The experiments were performed in duplicate and repeated once. FIC indices were calculated by the method of Eliopoulos & Moellering.[13] Synergy was defined as an FIC index of ≤0.5, indifference as an FIC index of >0.5 to ≤4 and antagonism as an FIC index of >4.

Selection of quinolone-resistant derivatives in vitro

Experiments were designed to test the tendency of levofloxacin to select resistant strains in liquid cultures. Large inocula (10^7–10^8 cfu/mL) of either WB4 or KR4 were exposed to stepwise increasing concentrations of antibiotics.[14] Series of tubes containing two-fold increasing concentrations of levofloxacin were incubated either with WB4 or KR4 (10^7–10^8 cfu/mL), as for MIC determination. After 12 h of incubation, 0.1 mL samples from the tubes containing the highest antibiotic concentration and still showing turbidity were used to inoculate a new series of tubes containing antibiotic serial dilutions. The experiments were performed over 12 cycles. The MIC was determined after each cycle.

In parallel, the same experimental protocol was used but ceftriaxone was added at a low concentration (0.03 mg/L corresponding to 1/16 MIC for the two strains) to the tubes containing serial dilutions of levofloxacin. After 12 h of incubation, the MIC of levofloxacin was determined as described above in tubes containing only levofloxacin.

Preparation of chromosomal DNA, PCR amplification and DNA sequence analysis

Chromosomal pneumococcal DNA was prepared as described previously.[15] PCR amplification of the *parC*, *parE*, *gyrA* and *gyrB* genes was performed according to a published method.[16] PCR amplification was performed with a GeneAmp PCR system 9700 apparatus (Perkin-Elmer). After amplification, PCR products were purified using a QIAquick PCR purification kit (Qiagen AG, Basel, Switzerland). Nucleotide sequencing for the PCR amplicons was carried out with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit according to the protocol of the manufacturer (Perkin-Elmer). An ABI PRISM 377 DNA sequencer was used for sequencing. All testing was performed in duplicate.

Results

One injection of ceftriaxone (125 mg/kg) led to peak CSF levels after 2 h: around 5.2 mg/L decreasing slowly to 3.0 mg/L at the end of the treatment period. During the total treatment period, ceftriaxone CSF levels remained far above the MIC. The peak and trough CSF/MIC ratios were 10.4 and 6, respectively. After one injection of 10 mg/kg levofloxacin, CSF levels peaked at 3.3 mg/L, decreasing slowly to 1.3 mg/L after 8 h.

The efficacy of the different regimens in rabbit meningitis are summarized in Table 1. In untreated controls, a slight increase in bacterial titres was observed over 8 h (+0.29 ± 0.10 $\Delta\log_{10}$ cfu/mL). Ceftriaxone produced only moderate bactericidal activity (–0.30 ± 0.09 $\Delta\log_{10}$ cfu/mL·h) without sterilizing the CSF of rabbits after 8 h (0 out of 9). Levofloxacin monotherapy produced significantly ($P = 0.0085$) higher antibacterial activity than ceftriaxone monotherapy, but managed to sterilize the CSF of only one rabbit. The combination regimen (ceftriaxone combined with levofloxacin) produced highly bactericidal activity, significantly superior compared with the monotherapies ($P < 0.01$ versus either monotherapy) and sterilized the CSF

Table 1. Single drug and combination therapy against penicillin-resistant *Streptococcus pneumoniae* WB4 in experimental meningitis

Antibiotic	*n*	Initial titre ($\log_{10}$ cfu/mL; means ± S.D.)	Killing rate ($\Delta\log_{10}$ cfu/mL·h; means ± S.D.)	Killing rate/8 h ($\log_{10}$ cfu/mL; means ± S.D.)
Controls	5	6.05 ± 0.50	+0.10 ± 0.50[a]	+0.29 ± 0.10[a]
Ceftriaxone	9	5.85 ± 0.45	–0.30 ± 0.09[b]	–2.20 ± 0.45[b]
Levofloxacin	9	6.11 ± 0.93	–0.45 ± 0.12[b]	–3.45 ± 0.76[b]
Levofloxacin + ceftriaxone	9	5.63 ± 0.20	–0.64 ± 0.07[b]	–5.33 ± 0.64[b]

[a] $P < 0.05$ versus all groups.
[b] $P < 0.05$ levofloxacin + ceftriaxone versus all monotherapies.

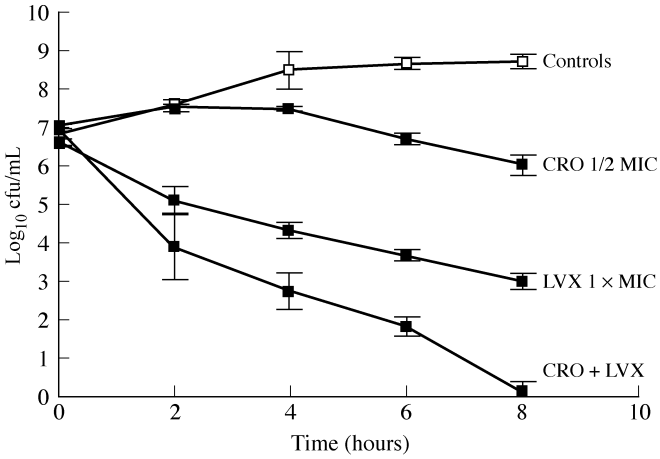

Figure 1. Killing rates of ceftriaxone (CRO 1/2× MIC), levofloxacin (LVX 1× MIC) and ceftriaxone combined with levofloxacin (CRO + LVX) for the penicillin-resistant strain WB4. Experiments were performed in triplicate, and killing rates are expressed as means ± S.D.

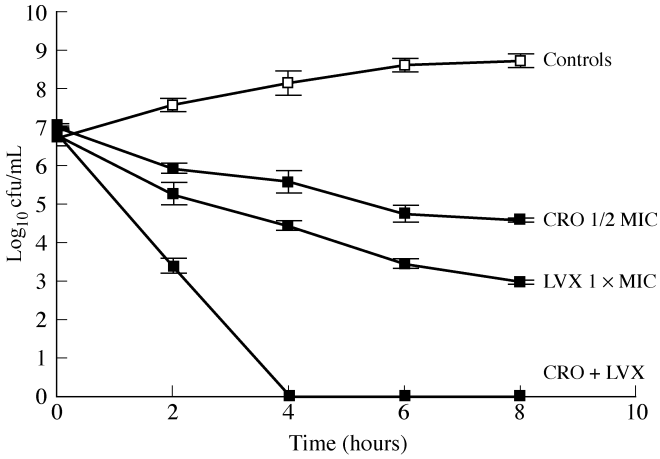

Figure 2. Killing rates of ceftriaxone (CRO 1/2× MIC), levofloxacin (LVX 1× MIC) and ceftriaxone combined with levofloxacin (CRO + LVX) for the penicillin-resistant strain KR4. Experiments were performed in triplicate, and killing rates are expressed as means ± S.D.

of eight out of nine rabbits at the end of the treatment period. In time–killing assays *in vitro*, antibiotic concentrations were chosen (ceftriaxone: 1/2× MIC and levofloxacin: 1× MIC) to produce only marginal intrinsic bactericidal activity in order to demonstrate potential synergy between the two compounds. Synergy was defined as the bactericidal effect of a drug combination significantly exceeding the sum of the bactericidal effects of each monotherapy.[13] Against WB4, ceftriaxone (1/2× MIC) produced a slight decrease in the viable cell count over 8 h (–0.8 $\log_{10}$ cfu/mL) (Figure 1). Levofloxacin monotherapy was more bactericidal with a decrease in bacterial titres around –3.9 $\log_{10}$ cfu/mL. The combination was highly bactericidal and acted synergistically (–6.9 $\log_{10}$ cfu/mL), sterilizing the cultures after 8 h. Against KR4, the synergistic activity of the combination was even more pronounced, sterilizing all cultures after 4 h (Figure 2). Ceftriaxone monotherapy was also more active against KR4 than against WB4 (–2.4 versus 0.8 $\log_{10}$ cfu/mL, respectively).

In addition, synergy between levofloxacin and ceftriaxone was demonstrated for both strains using the chequerboard method, with FIC indices of 0.25.

Based on a previous experimental setting,[17] levofloxacin-resistant mutants were selected in both WB4 and KR4 by sequential exposure to different levofloxacin concentrations over 12 cycles. In WB4 (Figure 3a), the MIC of levofloxacin increased to 8 mg/L after four cycles; after seven cycles, it increased to 64 mg/L and remained stable until the end of the experiment. In contrast, the MIC increased to a lesser extent with the addition of a low concentration of ceftriaxone (1/16 MIC). This latter concentration of ceftriaxone was the highest that allowed bacterial growth of pneumococcal cultures incubated with different concentrations of levofloxacin. After 12 cycles, the MIC was 16 mg/L. The increase in the MIC in the different treatment groups was confirmed by gene mutations: sequential exposure of WB4 to levofloxacin led to mutations in the target genes encoding for topoisomerase IV and gyrase ($Ser^{79}\rightarrow$Phe in *parC* and $Glu^{85}\rightarrow$Lys in *gyrA*). In the combination treatment, mutations in *parE* ($Asp^{435}\rightarrow$Asn) and *gyrA* ($Ser^{81}\rightarrow$Phe) were detected (see Table 2). In the second strain, KR4, the selection of levofloxacin-resistant mutants was comparable. In the monotherapy group, the MIC of levofloxacin increased to 8 mg/L after four cycles and to 64 mg/L after nine cycles (Figure 3b). Analagous to WB4, the addition of ceftriaxone (1/16 MIC) led to an MIC increase to 16 mg/L after 12 cycles. In the monotherapy group, two mutations in *parC* ($Ser^{79}\rightarrow$Phe and $Asp^{83}\rightarrow$Tyr) and one mutation in *gyrA* ($Glu^{85}\rightarrow$Lys) were detected. In the combination regimen, mutations in *parE* ($Asp^{435}\rightarrow$Asn) and *gyrA* ($Ser^{81}\rightarrow$Phe) were found. It is interesting to note that the addition of ceftriaxone at a low concentration (1/16 MIC) did not lower the MIC of levofloxacin. On the other hand, no cross-resistance

Table 2. Mutations in topoisomerase IV (*parC* and *parE*) and gyrase (*gyrA* and *gyrB*) genes before and after cyclic exposure to levofloxacin (LVX) alone or in combination with ceftriaxone (CRO) in two penicillin-resistant pneumoccocal strains (WB4 and KR4)

Strain	*parC*	*parE*	*gyrA*	*gyrB*
WB4	none	none	none	none
WB4 LVX	$Ser^{79}\rightarrow Phe$	none	$Glu^{85}\rightarrow Lys$	none
WB4 LVX+ CRO	none	$Asp^{435}\rightarrow Asn$	$Ser^{81}\rightarrow Phe$	none
KR4	none	none	none	none
KR4 LVX	$Ser^{79}\rightarrow Phe$ $Asp^{83}\rightarrow Tyr$	none	$Glu^{85}\rightarrow Lys$	none
KR4 LVX + CRO	none	$Asp^{435}\rightarrow Asn$	$Ser^{81}\rightarrow Phe$	none

between levofloxacin and ceftriaxone has been detected. The MIC of ceftriaxone was not affected by serial incubation of these two pneumococcal strains with levofloxacin (Table 3).

Discussion

In recent decades, pneumococci have developed several strategies to survive the pressure of numerous therapeutic modalities. They are able to resist β-lactam antibiotics by modifying the structure of bacterial cell-wall-synthesizing enzymes [also called penicillin-binding proteins (PBPs)] and the action of quinolones either by point mutations in the genes (*gyrA*, *gyrB*, *parC*, *parE*) or by activating efflux pumps to prevent intracellular accumulation of the drug. The emergence of vancomycin- and cephalosporin-tolerant strains, leading to treatment failure in cases of pneumococcal meningitis, has jeopardized the efficacy of this antibiotic combination, usually recommended as empirical treatment for meningitis. Furthermore, the emergence of quinolone resistance during therapy might undermine the use of quinolones as monotherapy for pneumococcal diseases.[4] The aim of this study was to evaluate a highly bactericidal regimen, which is a prerequisite for the treatment of pneumococcal meningitis, avoiding the risk of development of resistance at the same time.

The doses of ceftriaxone and levofloxacin were standard, mimicking levels achieved in humans. One injection of ceftriaxone led to CSF levels in rabbits in the range 5.2–3 mg/L, corresponding to levels measured in humans with bacterial meningitis.[18] CSF levels obtained with one injection of levofloxacin were slightly higher than those measured in humans after 500 mg twice daily (peak levels: 3.3 mg/L in rabbits versus 2.56–1.29 mg/L in humans).[19]

An interesting feature of this study was the efficacy of the combination regimen compared with the monotherapy in experimental meningitis (–5.33 $\Delta\log_{10}$ cfu/mL·8 h for the combination regimen versus –2.20 $\Delta\log_{10}$ cfu/mL·8 h for ceftriaxone and –3.45 $\Delta\log_{10}$ cfu/mL·8 h for levofloxacin), sterilizing the majority of the CSF of rabbits (eight out of nine). Ceftriaxone monotherapy produced killing rates similar to those published in previous studies against the same strain.[12,20,21] Compared with a previous study, levofloxacin monotherapy produced slightly higher killing rates, although the CSF peak levels were in the same range.[22]

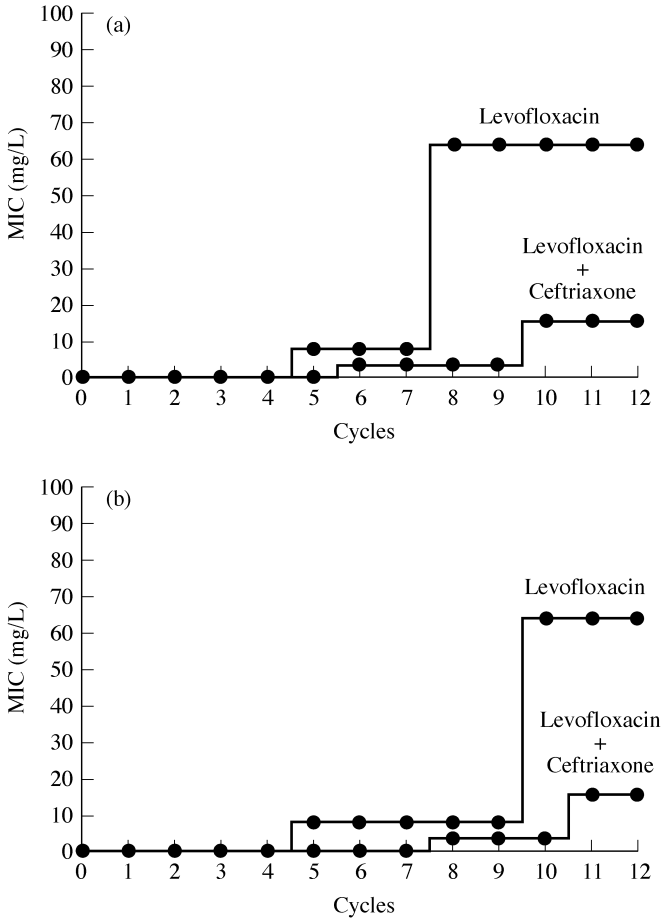

Figure 3. The selection of levofloxacin-resistant mutants of *Streptococcus pneumoniae* WB4 (a) and KR4 (b) exposed to stepwise increasing concentrations of levofloxacin alone or in combination with a sub-MIC concentration (1/16× MIC) of ceftriaxone.

In vitro, synergy was equal between ceftriaxone and levofloxacin in time–killing assays and with the chequerboard method with FIC indices of 0.25 for both strains.

An interesting aspect of this study was the effect of ceftriaxone, added at a low concentration (1/16 MIC), on the development of levofloxacin-induced resistance *in vitro*. The choice of the low concentration of ceftriaxone was based on results observed in the chequerboard experiments.

In cycling experiments with both strains, levofloxacin led to a stepwise increase of resistance until high-level resistance (MIC 64 mg/L) was reached at the end of the experimental period (12 cycles). The increase in resistance correlated with the sequential emergence of point mutations in genes encoding the two target enzymes, i.e. topoisomerase IV and gyrase. All mutations described in Table 2 have been mentioned in the literature.

In both strains, ceftriaxone prevented the emergence of mutations in *parC* but led to mutations in *parE* ($Asp^{435}\rightarrow Asn$). This mutation, which has been described in other isolates, selected after cycling with levofloxacin,[23] seemed to contribute to a lesser extent to the MIC increase. The effect of the β-lactam antibiotic, i.e. ceftriaxone added at a low concentration, on levofloxacin-induced resistance is not understood completely. Two explanations are conceivable: (i) the synergy between the two antibiotics might cause the bacterial popu-

Table 3. MICs of levofloxacin (LVX) and ceftriaxone (CRO) alone and levofloxacin in combination with a subinhibitory concentration of ceftriaxone for two penicillin-resistant strains (WB4 and KR4)

	MIC (mg/L)					
	WB4	WB4 LVX	WB4 LVX + CRO	KR4	KR4 LVX	KR4 LVX + CRO
LVX	1	64	16	1	64	16
LVX + 1/16 MIC CRO	1	64	16	1	64	16
CRO	0.5	0.5	0.5	0.5	0.5	0.5

WB4, quinolone-susceptible but penicillin-resistant parent pneumococcus (MIC 4 mg/L).
WB4 LVX, levofloxacin-resistant derivative selected by passages on this drug.
KR4, quinolone-susceptible but penicillin-resistant parent pneumococcus (MIC 4 mg/L).
KR4 LVX, levofloxacin-resistant derivative selected by passages on this drug.
WB4 LVX+CRO or KR4 LVX+CRO, same as above, but cycled in the presence of subinhibitory concentrations of ceftriaxone.

lations to decrease below a critical threshold for longer, i.e. below 10^6–10^7 cfu/mL where mutations occur; (ii) the combined antibacterial effect of antibiotics interfering with different targets (PBPs or gyrase and topoisomerase IV) might impede the development of mutations in the microorganism. The last hypothesis is probably the more unlikely because the MIC of levofloxacin was not affected by the addition of ceftriaxone (see Table 3).

The observation that antibiotics interfering with the cell wall synthesis might influence quinolone-induced resistance in pneumococci is not new. In the same experimental setting, we have shown previously that the addition of vancomycin reduced ciprofloxacin- and trovafloxacin-induced resistance in the strain WB4.[17]

In summary, we have demonstrated that a combination of ceftriaxone and levofloxacin was very efficacious in experimental pneumococcal meningitis and reduced the risk of quinolone-induced resistance. This combination might be used in the future as empirical treatment in bacterial meningitis, as an alternative to the recommended regimen based on ceftriaxone and vancomycin.

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